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Supplementary Materials for

Accessing neuroinflammation sites: Monocyte/neutrophil-mediated drug delivery for cerebral ischemia

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This PDF file includes:

Supplementary Materials

Supplementary Materials and Methods

Cell culture and Animal

THP-1 was cultured in RPMI-1460 media supplemented with 2% fetal bovine serum (FBS). For activation, THP-1 was stimulated every other day for 12 days with human interleukin-2 (hIL-2), a cytokine that is known to induce pro-adhesive molecule expression in monocytes (***41)***. The activation was verified by the formation of cell clusters 10-12 days after the treatment. HL-60 cell line was cultured in Iscove's modified Dulbecco minimum essential medium (IDMEM) with 10% FBS, and the differentiation into polymorphonuclear-like cells was activated by adding 1.3% DMSO 96h before experiment as previously reported (*30*). HBMEC was cultured on upper chamber of Transwell insert of purified collagen with endothelial cell medium containing 1% endothelial cell growth supplemented with 5% FBS. PC12 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated FBS and l-glutamine (2 mM).

Synthesis of cRGD-conjugated DSPE-PEG

Activated DSPE-PEG (DSPE-PEG-NHS, PEG-3400) was conjugated with cRGD. Briefly, cRGD and DSPE-PEG-NHS at a molar ratio of 2:1 were separately dissolved in DMF. The cRGD solution was then added dropwise to the DSPE-PEG-NHS solution with gentle stirring. N-methylmorpholine was added to the mixture to adjust the pH to 8.0. The reaction was performed at room temperature for 6h and monitored by HPLC until the disappearance of the peak of cRGD. The formation of cRGD-PEG-DSPE was confirmed by MALDI TOF with the yield of 76.5% (fig. S1). Excessive cRGD peptide was removed by dialysis against water (molecular weight cut-off of 2000).

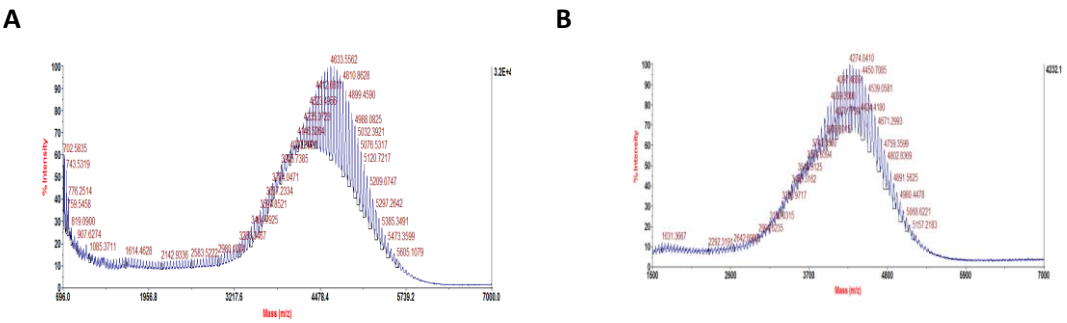


Fig. S1. The synthesis of cRGD peptide with PEG-DSPE assayed by MALDI-TOF. (A) DSPE-PEG-NHS with average molecular weight of 4274. (B) cRGD-PEG-DSPE with average molecular weight of 4633.

Preparation and characterization of liposomes

PLs were prepared with SPC, DSPE-PEG (PEG-3400), DSPG and cholesterol using a modified reverse-phase evaporation method. Briefly, phospholipids (10mg/ml) and cholesterol, including 5 mol% DSPE-PEG of total lipid, were dissolved in 15ml chloroform and 5ml phosphate buffer (pH7.4, 150mM). The resulting two-phase system was subjected to bath-type sonication and then placed in a rotary evaporator under reduced pressure for at least 12h until a homogeneous suspension of PLs were obtained.

For the preparation of cRGDLs, 5% cRGD-PEG-DSPE was used in the lipid formulation instead of DSPE-PEG. The suspension was then subjected to ultrasonic treatment for 2 min at 500 watts to obtain desired particle size.

For fluorescence liposomes, hydrophilic fluorescence marker (Cy5) was added into PBS and hydrophobic marker (NBD-PE, C6 or Dir) was dissolved in chloroform. Excessive fluorescence marker was removed by Sephadex G-50 with size exclusion chromatography.

For the preparation ER-cRGDLs or ER-PLs, ER was added into lipid formulation (lipid to drug 10:1) and dissolved in chloroform as described above. The EE was determined by size exclusion chromatography and ultra-performance liquid chromatography method. The equation for calculating EE was the following:

$$EE=W_{interior}/W_{total}\times 100\%$$

$W_{interior}$ represents the intraliposomal content of ER, and W_{total} represents the total content in the liposomal suspension when Triton-100 was added to the suspension.

We performed a complete characterization for all the liposomes used in the study. Nanoparticles properties such as particle size, zeta potential, morphology and drug/dye loading efficiency were measured in a time course manner (fig. S2, table S1). Samples were diluted 100 times with PBS (pH7.4, 150mM) for particle size, zeta potential with dynamic light scattering at room temperature. Liposomes were diluted 1000 times and dropped onto the copper gird, followed by 1% uranyl acetate stain for observation under a high-resolution transmission electron microscope (TEM). For entrapment efficiency determination, free dye or free drug was

separated by Sephadex G-50 column and determined by HPLC or fluorospectrophotometer to calculate the EE as mentioned above.

Table S1. Characterization of all liposomes used in the study.

Liposomes	**EE (%)**		**Average particle size (nm)**		**Zeta potential (mV)**	
	0day	7 day	0d	7 d	0d	7 d
PLs			150.3	155.6	-26.8	-25.1
cRGDLs			150.9	159.9	-26.6	-27.8
ER-PLs	74.5	74.3	151.1	158.5	-26.3	-25.7
ER-cRGDLs	74.3	74.2	152.2	153.1	-26.6	-26.0
C6-PLs	88.6	87.9	149.7	151.2	-25.9	-24.8
C6-cRGDLs	89.5	88.6	148.2	153.5	-27.7	-24.3
Cy5-PLs	70.3	68.1	151.1	153.1	-26.3	-23.5
Cy5-cRGDLs	71.0	70.2	152.7	155.2	-25.2	-24.8
DiR-PLs	85.7	85.0	152.9	156.7	-25.9	-25.0
DiR-cRGDLs	84.1	83.2	152.3	155.9	-25.7	-24.3

*EE of Cy5 and NBD, respectively. EE, average particle size and zeta potential of all samples were determined at 0 and 7 days.

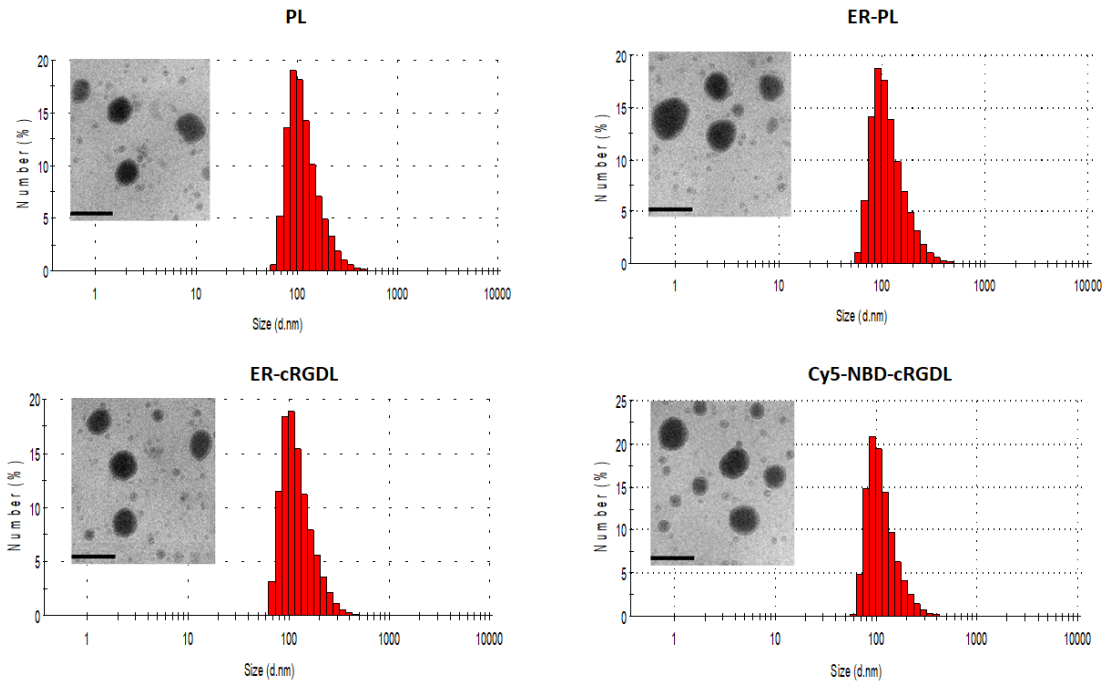


Fig. S2. TEM photos and size distribution of various liposomes.

The bar in TEM photo represents 200nm. The size distribution was assayed by dynamic light scattering.

Dispersion characterization of liposomes in different medium

Cell culture (DMEM) with 10% fetal bovine serum, 10% serum, 30% serum and 50% serum (diluted with PBS) were conducted in 24-well plates or 10 mL test tubes to value the dispersion characterization of PLs and cRGDLs in different media. 100 μL of PLs or cRGDLs were added to 1 mL DMEM or 1 mL serum of different concentration, following gentle mixture and 30 min incubation. Samples were taken out from three positions in the middle of the suspension and three positions at the bottom of the suspension at 10 min and 30 min, respectively. Then samples were diluted 10 times with corresponding medium for particle size determination and derived count rate was recorded with dynamic light scattering at room temperature (figs. S3 and S4).

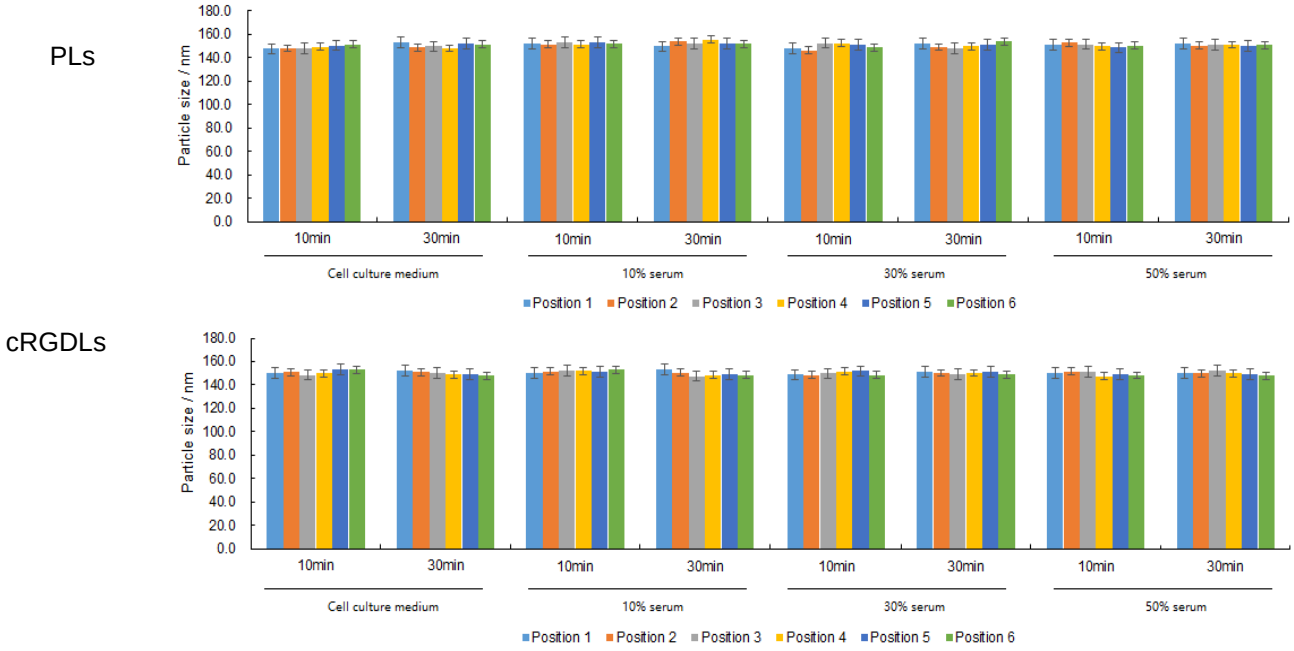


Fig. S3. Particle size of PLs/cRGDLs in different positon of the suspension or different medium by dynamic light scattering at different time.

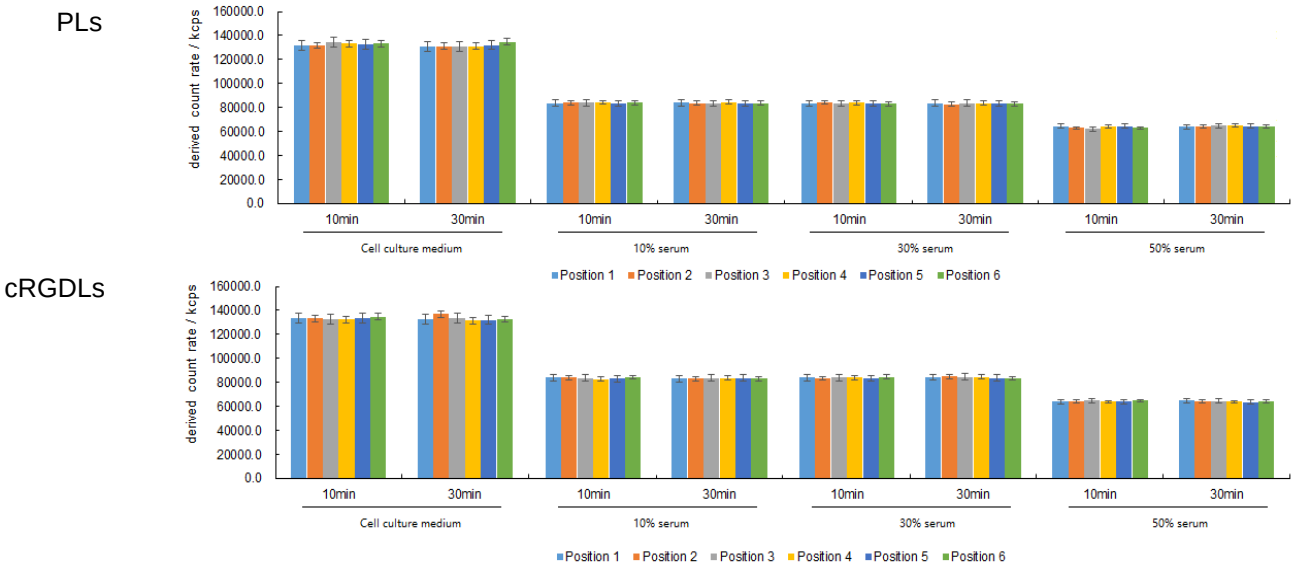


Fig. S4. Derived count rate of PLs/cRGDLs in different positon of the suspension or different medium by dynamic light scattering at different time.

Isolation of M/Ns by density gradient centrifugation

The whole blood was extracted from the cardiac puncture using Histopaque® density gradient reagents. 3mL Histopaque 1119 was added to a 15 mL tube, and 3 mL Histopaque 1077 was gently pipetted on top of it. The

whole blood was layered carefully over the upper gradient, and was centrifuged 400xg, 30 mins under room temperature, without using a brake. The neutrophils were collected using pipet from the interface of Histopaque 1077 and 1119 layers, and monocytes between the upper gradient and the plasma. The cells were washed with PBS twice, respectively.

Human monocytes or neutrophils were isolated from fresh human peripheral blood within 2 h of collection by the density gradient centrifugation method. To verified the purity, CD14 and CD15 were determined for monocytes; CD 45, CD15/CD16, CD11b/CD66b were assayed for neutrophils. All the purity of M/Ns used in this study is above 95%.

Immunofluorescence histochemistry

Immunofluorescence staining of cerebral vessels, neurons, microglia and NeuN was performed on 20μm coronal section. Alexa Fluor® 594 Goat Anti-Rabbit, Alexa Fluor® 405 donkey anti-mouse secondary antibody and Alexa Fluor® 405 Goat Anti-Rabbit were used for fluorescence imaging. Sections were observed with a confocal laser scanning microscope.

Focal cerebral I/R models in rats

Focal brain ischemia was induced by MCAO as described previously (*36*). In brief, rats were anesthetized by intraperitoneal injection of 10% chloral hydrate at a dose of 400 mg/kg (0.5mg/kg for nude mice). The left middle cerebral artery was occluded for 1.5 h followed by reperfusion. Rectal temperature was recorded and maintained between 36.5 °C and 37.5 °C throughout the surgical procedure and up to 1.5 h after reperfusion. Regional cerebral blood flow was measured by laser Doppler flowmetry before, during and after MCAO, as well as before the sacrifice of the rats. The mice of the sham-operated vehicle underwent the same above-mentioned protocol except for MCAO.

Balance beam test

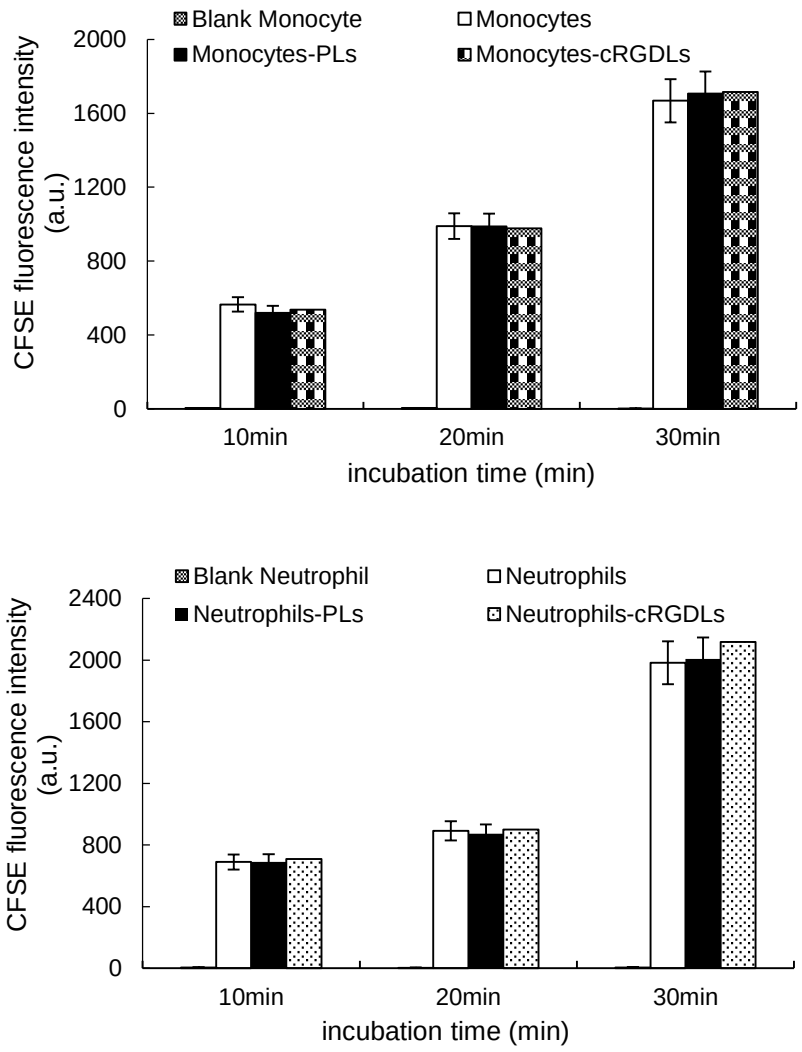
The rats took the beam-walking test in order to test its sensorimotor integration. An 80-cm-long, 2.4-cm-wide beam was placed 10 cm above the ground, on which the rats were placed to walk. A 5-point scale was adopted in the test as follows (*36*): 0, the rats were able to balance and walk on the beam using its forelimbs symmetrically; 1, the rats were able to balance and walk on the beam using its unaffected limb preferentially;

2, the rats were able to balance and walk on the beam mostly relying on the unaffected limb; 3, the rats were not able to balance on the beam once moved; 4, the rats felled off the beam immediately. The average scores were calculated for statistical analysis. The higher score represented a worse functional outcome.

Morris Water Maze

The MWM test was performed in a 1.5 m diameter circular pool that was filled to a depth of 30cm with 20±1℃ tap water (37). A black 10 cm diameter columnar escape platform was placed 1.5 cm below the water surface. Training was consisted of 4 trials per day for 5 consecutive days, to find a hidden platform. During training, the platform was placed in a fixed quadrant of the pool and the rat was placed in each of the 4 quadrants at the start of trial, positioned facing the wall. If the rats did not reach the hidden plat form within 1min, it was placed on the platform to rest for 15 s. The initial quadrant placement was consistent on each day of testing. Latency to find the platform, swimming speed were measured. On day 6^{th}, the platform was removed, and the rats were allowed to swim in the water for up to 60s, starting from the same quadrant and facing the wall. The swimming track, time spent in each quadrant and speed were recorded in each trial.

A



B

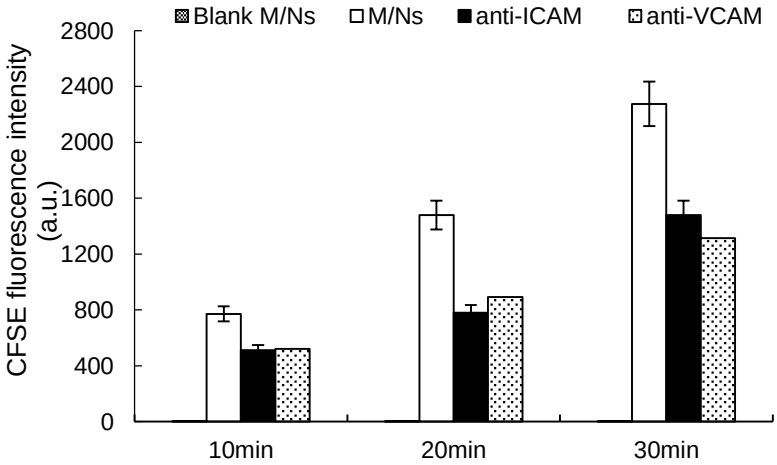
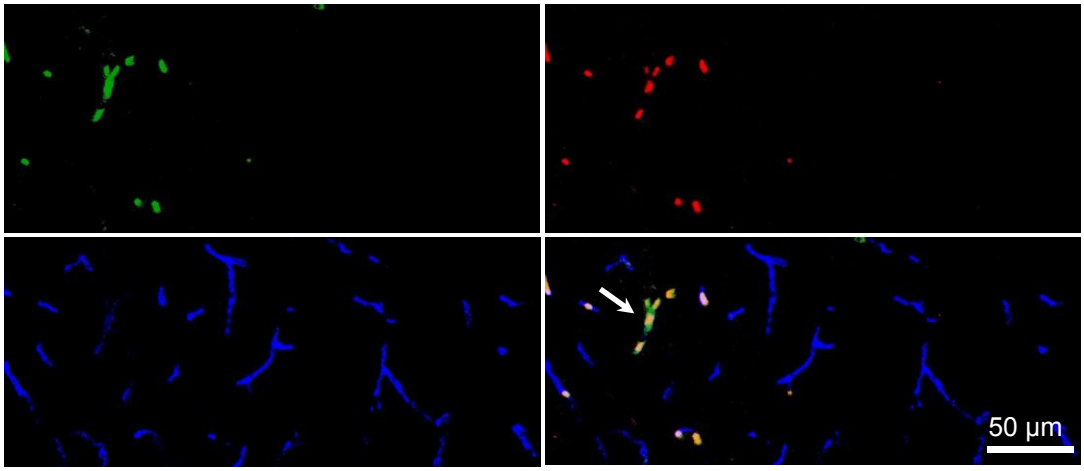


Fig. S5. The influence of liposomes and antibodies on the migration of M/Ns. (A) The fluorescence intensity of migrated M/Ns with or without PLs and cRGDLs treatment. (B) The inhibition of ICAM and VCAM on the M/Ns migration.

Cy5-NBD-PLs



Cy5-NBD-cRGDLs

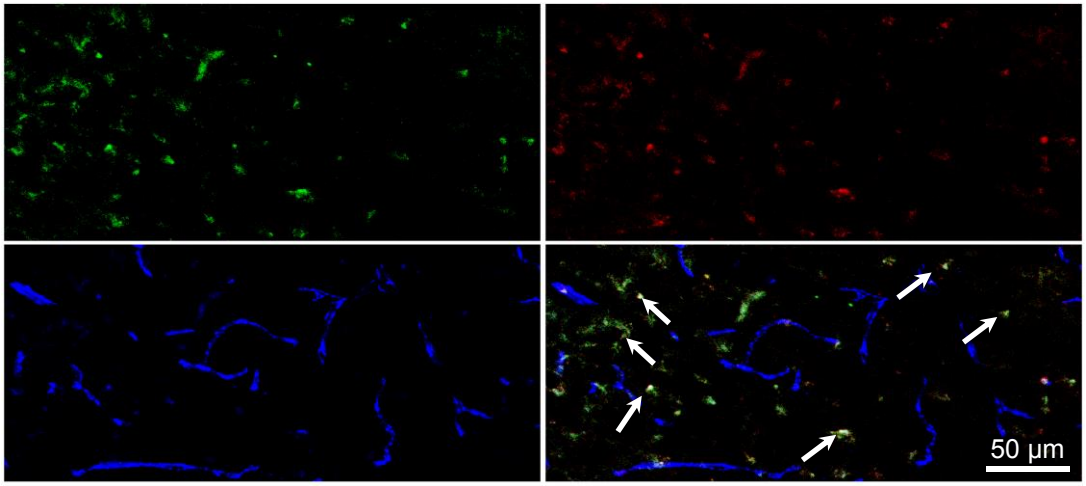


Fig. S6. Location of liposomes in ischemic brains of I/R rats 12 hours after reperfusion. Confocal microscope images of co-location of Cy5-NBD-PLs/Cy5-NBD-cRGDLs with cerebral vascular tree which were labeled with vWF rabbit anti-rat antibody and secondary antibody of Alexa Fluor® 405 Goat Anti-Rabbit. Green: NBD-PE marker; Red: Cy5 marker; Blue: vWF.

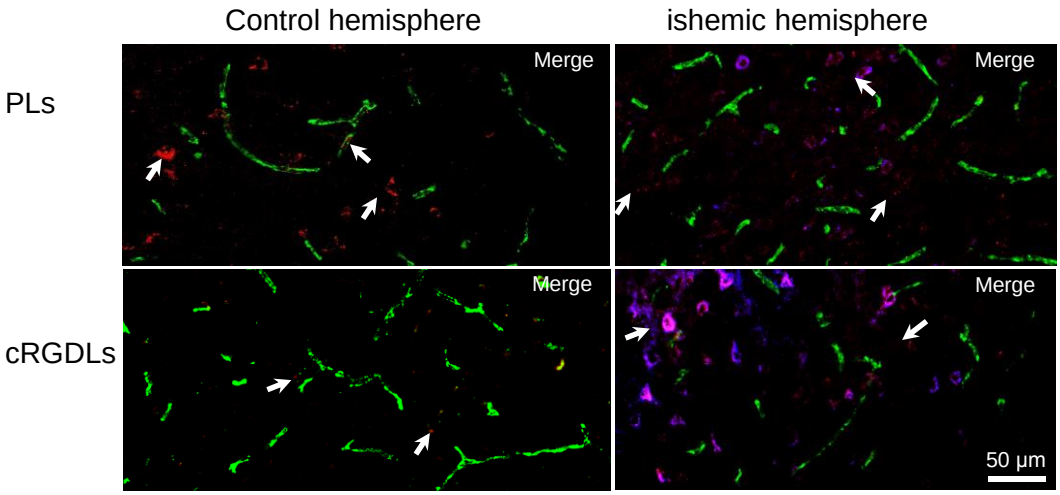


Fig. S7. Confocal microscope images of comigration of Cy5-PLs/Cy5-cRGDLs with M/Ns across cerebral vessels in the nonischemic hemisphere 24 hours after reperfusion. Blue: M/Ns stained with cell tracer CFSE; Red: Cy5-PLs or Cy5-cRGDLs; Green: cerebral vessels label with vWF rabbit anti-rat antibody and secondary antibody of Alexa Fluor® 594 Goat Anti-Rabbit.

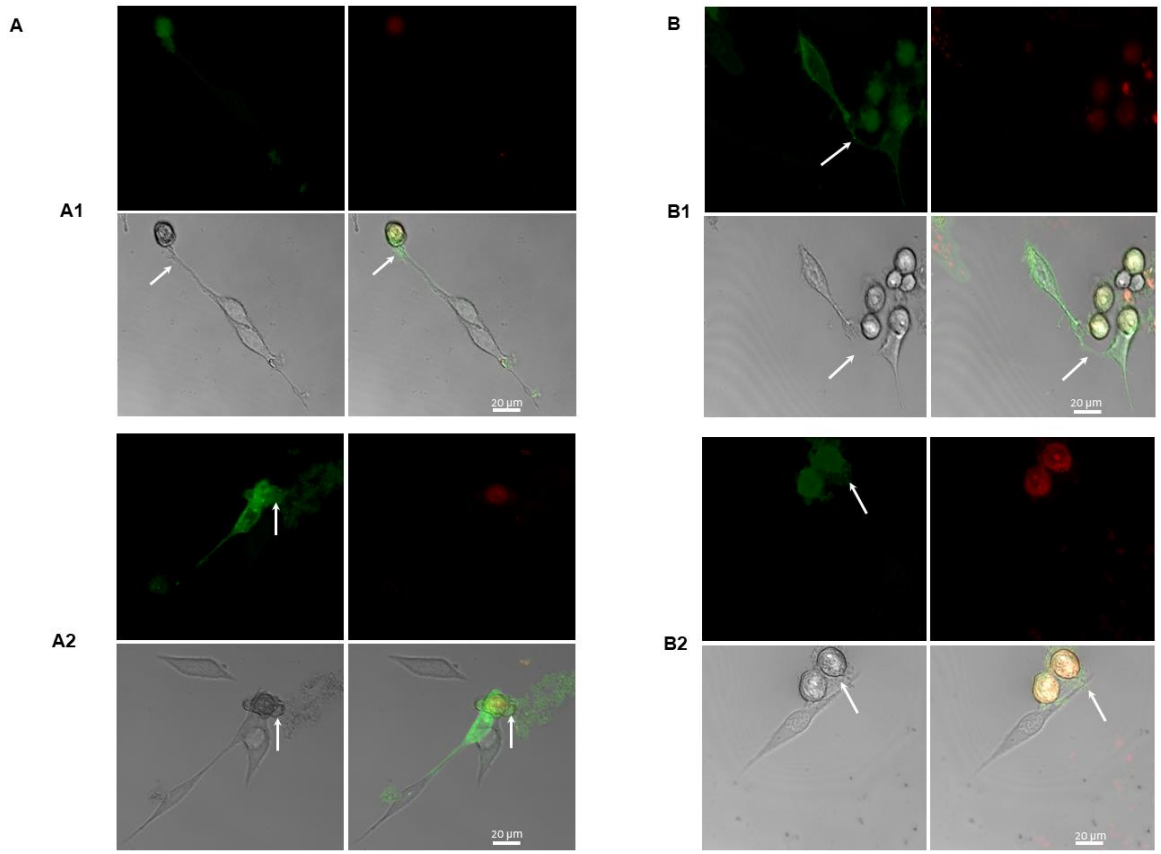


Fig. S8. Transfer mechanisms of the liposomes from immune cells to neuron cells. Transfer mechanisms of the liposomes from (A) THP-1 or (B) HL-60 to PC12.

Confocal microscope images of cell membrane transfer (A1, white arrow), cell deformation (A2, white arrow), bridge between cells (B1, white arrow), and exosome secretion (B2, white arrow). Green: THP-1 or HL-60 strained with DiI; Red: Cy5-cRGDLs; PC12 wasn't stained.

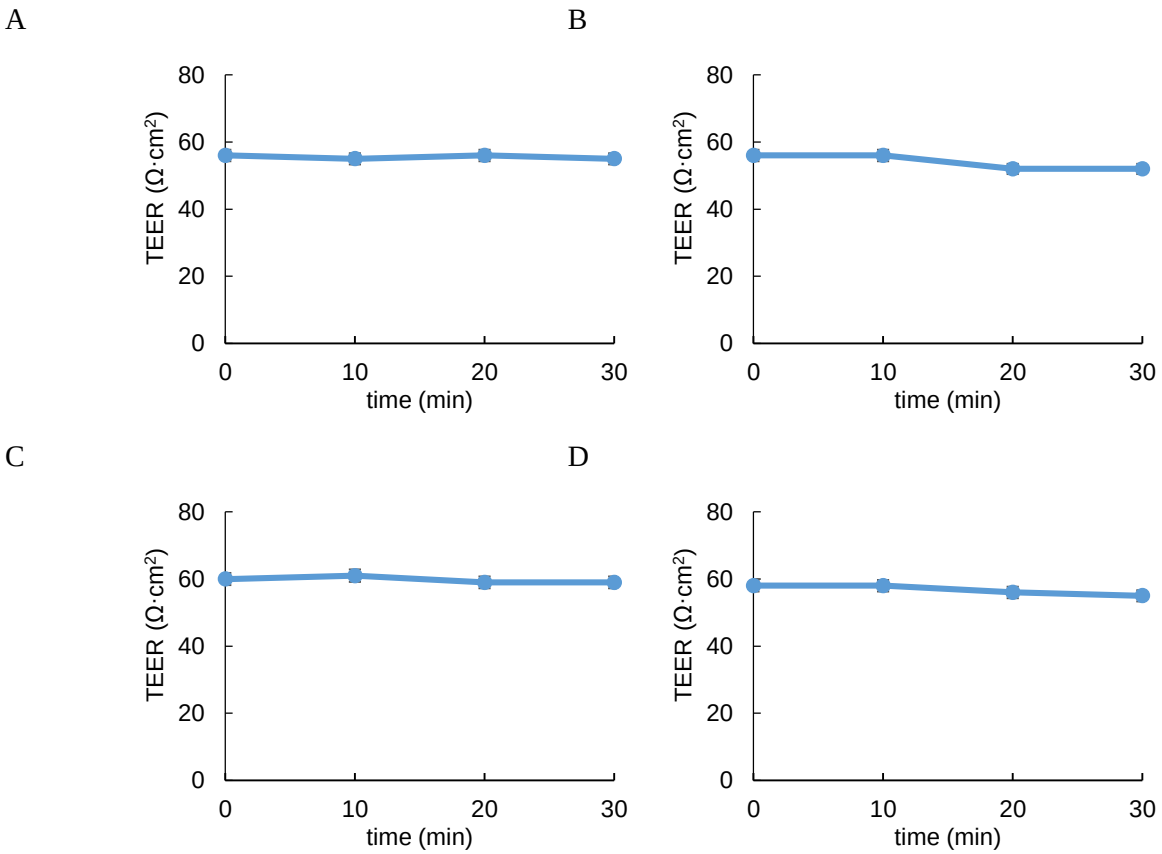


Fig. S9. TEERs of HBMEC in comigration and inhibition assay.

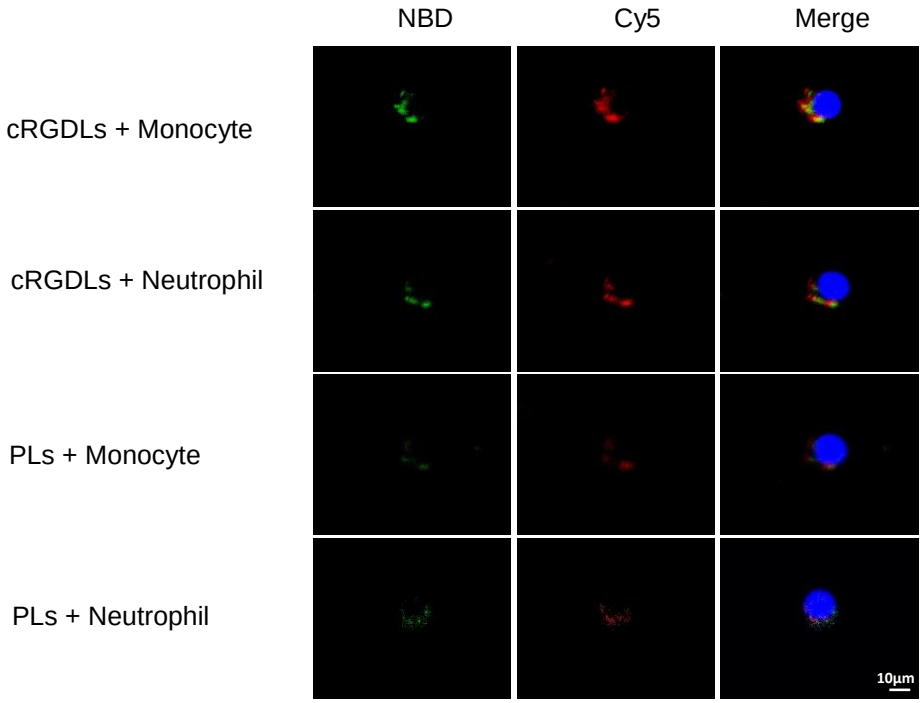


Fig. S10. Confocal microscope images of cellular location of Cy5-NBD-cRGDLs in M/Ns.

Table S2. The pharmacokinetics parameters of I^{125}-labeled PG.

		I^{125}-PG	I^{125}-PG-PLs	I^{125}-PG-cRGDLs
$AUC_{(0-\infty)}$	DPM/L*h	253967.7	334236.4*	524901.46$^{**\#\#}$
$t_{1/2}$	h	3.131237	3.462737	5.4023672$^{**\#\#}$
CL	L/h/kg	630001.5	478703.1**	304819.12$^{**\#\#}$

$^{**}P<0.01$, $^{*}P<0.05$, compared with I^{125}-PG; $^{\#\#}P<0.01$, $^{\#}P<0.05$, compared with I^{125}-PG;